

## THE ROLES OF HEMOCYTES IN TANNING DURING THE MOLTING CYCLE: A HISTOCHEMICAL STUDY OF THE FIDDLER CRAB, *UCA PUGILATOR*

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### ABSTRACT

Histochemical data support the previous biochemical finding that the blood is a major site for the production of proteinaceous and diphenolic substances for tanning of the cuticle in the fiddler crab, *Uca pugilator*. Five types of hemocytes are described. Specifically in tanning, the hyaline cells (cystocytes) appear responsible for the production of diphenolic tanning agents whereas the granulocytes synthesize the proteins involved. Other types of hemocyte may be transitional forms involved in clotting (intermediate cells). Various histochemical reactions for each type of hemocyte and the cuticle are recorded throughout the molting cycle, and appear cyclic. The data suggest there is hormonal control of the cyclic events during the tanning process.

### INTRODUCTION

At least in some arthropods, sclerotization consists of two major processes: (1) the biosynthesis of tanning agents (N-acetyldopamine and N-acetylnoradrenalin) from their amino acid precursors (tyrosine and phenylalanine), and (2) the subsequent incorporation of the newly formed tanning agents into the cuticle (Brunet, 1965; Koeppel, 1971; Vacca and Fingerman, 1975a, b). In the cockroach, the synthesis of the tanning agent, N-acetyldopamine, begins within the hemocytes (Whitehead, 1969). However, in crustaceans, the synthesis site of the tanning agents remains unknown. Crustaceans, like insects, maintain high metabolic pools of free amino acids within the hemolymph (Awapara, 1962; Florkin and Schoffeniels, 1965). In the hemolymph of the crab, *Carcinus maenas*, most of the free amino acid pool is concentrated within the hemocytes (Evans, 1972). The blood cells, although they provide only 1% of the total blood volume, contain 58% of the total free amino acid concentration. In this way, the blood cells maintain a steep gradient against the serum; but the purpose of this gradient remains obscure. Presumably some of the free amino acids could serve as precursors for tanning agents and their protein carriers.

Early workers regarded one type of crustacean hemocyte, the granulocyte, as a carrier of metabolites (Tait and Gunn, 1918). However, more recent evidence supports other functions also, including phagocytosis, wound agglutination, blood coagulation, parasitic encapsulation, basement membrane formation, and storage of glycoproteins (George and Nichols, 1948; Dumont *et al.*, 1966; Bang, 1967; Wood and Visentin, 1967; Strutman and Dolliver, 1968; Busselen, 1970; Wood *et al.*, 1971; Ravindranath, 1980). On the other hand, these data fail to explain why the clotting ability of the blood is minimal at ecdysis, precisely when the soft-shelled animal is most susceptible

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Abbreviations: Az-Eo, azure-eosin; DAS, diazosulfanilic acid; DAS-AzA, diazosulfanilic acid pH 1 azure A; DOPA, dihydroxyphenylalanine; FeII, ferrous iron; FeIII, ferric iron; NQS, beta-naphthoquinone-4-sodium sulfonate; PAS, periodic acid-Schiff; PCB, post-coupled benzylidine; RNA, ribonucleic acid.

to injury and infection (Bang, 1967; Levin, 1967; Strutman and Dolliver, 1968). Since the hemocytes do not clot well during ecdysis, they may be involved with yet another and more important function during this period, namely tanning.

Reportedly, cyclic fluctuations occur during the molting cycle in: (a) the enzymatic activity of blood phenoloxidase (Pinhey, 1930; Declair and Vercauteren, 1965; Summers, 1967); (b) the numbers of circulating hemocytes (Bruntz, 1907; Kollman, 1908; Marrec, 1944); and (c) the appearance of carrier proteins which transport tanning agents from the hemolymph into the cuticle at ecdysis (Vacca and Fingerman, 1975a, b). These cycles suggest that the hemocytes of crustaceans may have a special function which is intimately associated with the tanning process. That the hemocytes can penetrate the epithelium and synthesize protein during the secretion of the proecdysial cuticle in the crayfish *Orconectes limosus* (Keller and Adelung, 1970) further implies that they are involved in the tanning process. The present investigation explores this possibility in the fiddler crab, *Uca pugilator*, by a histochemical study of the hemocytes during the molting cycle. The histochemical reactions of the developing exoskeleton are correlated.

#### MATERIALS AND METHODS

All observations were made on fiddler crabs (*Uca pugilator*) during various stages of the molting cycle. Stock male and female fiddler crabs were maintained individually in finger-bowls containing enough artificial sea water (Instant Ocean, Aquarium Systems, Inc.) to cover the bottom 1 cm deep. The water was changed every 2-3 days after the animals were fed a few flakes of oatmeal. Crabs were induced into a precocious proecdysial period and eventual ecdysis as previously described (Vacca and Fingerman, 1975a, b) by removing both eyestalks (Brown and Cunningham, 1939) or by autotomizing several legs (Skinner and Graham, 1972; Fingerman and Fingerman, 1974). The intermolt crabs were intact specimens that had undergone ecdysis (induced by limb removal) and limb regeneration at least 1 month prior to use.

Stages of molt were determined according to Guyselmann (1953). Proecdysial animals were selected from eyestalkless or autotomized crabs. They showed external evidence of apolysis, a bluish gray opalescence on the carapace. Forty-four specimens were selected at different stages in the molting cycle including ecdysis, various times of postecdysis (5, 10, 24 and 48 h), proecdysis, and intermolt. The crabs were fixed *in toto* either by injection of, or immersion in various fixatives including 10% neutral phosphate-buffered formalin and 6% neutral phosphate-buffered glutaraldehyde to which 6% NaCl was added; formalin-acetic acid-salt (10%:5%:5%); chloroform:methanol (2:1); and Barnett and Bourne silver fixative (Lillie, 1965).

The crabs were bisected to allow rapid entry of the fixatives, and were fixed for 24 hours. After a thorough washing, they were dehydrated in graded alcohols, and cleared in xylene. Tissues were then embedded in paraffin *in vacuo*.

Tissue sections (6-8  $\mu\text{m}$ ) were stained with azure-eosin (Az-Eo), pH 4.5, and examined for numbers and types of blood cells. The extinction coefficient of basic dye uptake by the hemocytes was determined with toluidine blue 0 (0.1%) at pH 1 through 3.

Other histochemical tests included: the periodic acid-Schiff (PAS) reaction for the identification of 1,2-glycols (Mowry, 1963); black Bauer and black periodic techniques for aldehyde detection (Lillie, 1965); and Sudan black B for the localization of lipids (Lillie, 1965).

In conjunction with these procedures, various blockades were used. Acetylation was accomplished after 3 hours at 60°C in a 2:3 mixture of acetic anhydride: pyridine

(Barka and Anderson, 1963), to distinguish lipid from other PAS-reactive substances. Deacetylation was performed by immersing tissue sections in ammonium hydroxide:ethanol (1:4) for 24 hours (Lillie, 1965). Incubation in saliva (1–3 h) was used to identify glycogen. To distinguish bacteria from other intra- and extracellular inclusions, ribonucleic acid (RNA) was extracted by incubating tissue sections in KOH (1% in 70% ethanol, 15–20 min.).

Several diazotized dyes were prepared for the demonstration of proteins and phenols (Lillie, 1965). These included: diazosafranin, pH 3.2 for serotonin (Lillie *et al.*, 1973a), or pH 7.8 for proteins; and diazosulfanilic acid, followed by pH 1 azure A (DAS-AzA), for norepinephrine or another primary catecholamine (Lillie *et al.*, 1973b). Lack of extraction of the colored tissue sites by acid (0.1 *N* HCl for 24 h at room temperature) verified azo-coupling.

Blocking procedures were used in conjunction with the localization of phenols. Oxidation was carried out with periodic acid (1%, 30 minutes); reduction with 5% sodium dithionite (2 or 4 two-hour incubations). Ferrous chloride (FeCl<sub>2</sub>, 0.1 *M*, 2 h), freshly prepared by the method of Lillie *et al.* (1971), was used to block histidine staining by the DAS-AzA technique, was previously demonstrated in mammalian erythrocytes (see Lillie *et al.*, 1973b, c).

Indole derivatives were visualized by the post-coupled benzylidine (PCB) reaction (Glennner and Lillie, 1957). The beta-naphthoquinone-4-sodium sulfonate (NQS) method of Lillie *et al.* (1971) was used to demonstrate sites rich in arginine. The Morel-Sisley procedure for the demonstration of tyrosine was also applied (Lillie, 1965). The reaction for tyrosine was blocked by pretreatment (6 h at room temperature) with tetranitromethane (0.1 ml in 10 ml pyridine to which 20 ml 0.1 *N* HCl was added).

To demonstrate amino groups, slides were mordanted for two hours in FeCl<sub>2</sub>, then stained with neutral hematoxylin, with and without prior deamination. Deamination was accomplished over a 24 hour period at 4°C in a mixture of 14% sodium nitrite in 2 *N* acetic acid.

Ferric ferricyanide was used to identify reducing sites. To distinguish phenolic sites from iron reaction, sections were reacted with acid ferri- and ferrocyanide. To differentiate between sites of reduction and oxidation respectively sections were first mordanted in FeCl<sub>2</sub> (0.1 *M*; 2 h), then reacted with acid ferri- and ferrocyanide.

Sections were incubated in acid silver (0.1 *M* AgNO<sub>3</sub> in 0.01 *M* acetate buffer, pH 5.0) in the dark (24 h at room temperature) to demonstrate further the presence of reducing substances (Lillie, 1957). Additionally, ammoniacal silver procedures were applied to the tissue sections for 10 minutes and 24 hours in the dark at room temperature (Lillie, 1965).

Several procedures were used to localize copper. These included Clara's (Mallory's neutral) hematoxylin (Lillie, 1965) and ammoniacal rubanic acid, with and without mordanting in a copper sulfate solution (2.5% in 50% alcohol for 2 h).

## RESULTS

During all stages of the molting cycle, two main types of blood cells could be distinguished histochemically by the presence or absence of acidophilic cytoplasmic granules (Az-Eo; Figs. 1A, 2A): (a) large hemocytes containing numerous acidophilic (eosinophilic) granules in an abundant acidophilic cytoplasm were recognized as granulocytes and (b) smaller agranular cells exhibiting a scanty pale basophilic cytoplasm around an intensely basophilic nucleus were identified as hyaline cells (also known as cystocytes). On rare occasions, a third type of blood cell could be seen (Fig. 2) which seemed to be an "intermediate" or transitional type. It resembled the hyaline

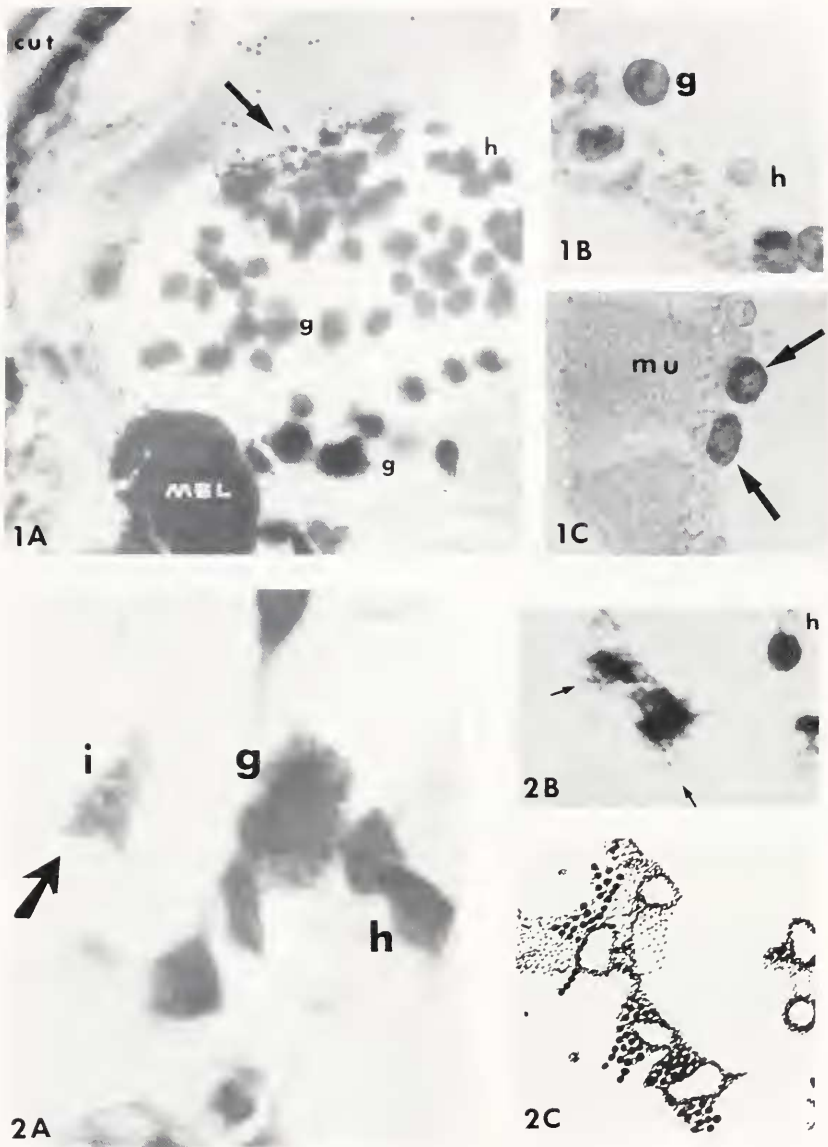


FIGURE 1. Two main types of hemocyte can be identified in the blood of *Uca*: granulocytes (g) and hyaline cells (h). In Figure 1A these hemocytes aggregate in great numbers near the epidermis and soft cuticle (cut) of a crab fixed in a buffered glutaraldehyde-salt fixative. Granulocytes and hyaline cells appear scattered within the eosinophilic serum which contains numerous granules (arrow). Certain granules exhibit basophilia; others exhibit acidophilia. Az-Eo, pH 4.5. MEL, melanophore.  $\times 430$ . Figure 1B shows the positive reaction for arginine in the granulocytes (g) and the negative reaction in the hyaline cells (h). NQS.  $\times 600$ . Figure 1C demonstrates reducing substances in small (immature?) granulocytes (arrows) found deep within the hemocoel. Ferric ferri cyanide. mu, muscle.  $\times 600$ .

FIGURE 2. Besides hyaline cells and granulocytes, a third type of hemocyte (intermediate or transitional type) can be seen within the hemocoel. In Figure 2A a specimen fixed in formalin:acid:salt during late intermolt, the intermediate type (transitional cystocyte) hemocyte (i, arrow) approximates the size of the hyaline cell (h) and exhibits tiny unstained granules within a less extensive cytoplasm than the granulocytes (g). Az-Eo, pH 4.5.  $\times 900$ . Figure 2B shows intermediate cells (arrows) releasing tiny proteinaceous granules which have azo-coupled with DAS. The hyaline cell (h) contains diphenols. The hemocytes appeared in a specimen fixed 10–15 hours postecdysis in buffered formalin-salt.  $\times 600$ . Figure 2C represents a diagrammatic interpretation of the intermediate cells rupturing and releasing their granules, thereby forming a cytoplasmic network which may function in clotting.



cell in size and nuclear:cytoplasmic ratio, but it contained a number of cytoplasmic granules like the granulocyte. However, the granules were smaller than those observed in the granulocyte and were refractory to staining with acid and base dyes; also they occurred within an unstained cytoplasm. Two additional types of granule-containing (transitional?) cells, large and small granular cells contained swollen granules which were discerned by other histochemical procedures (Figs. 3 and 4).

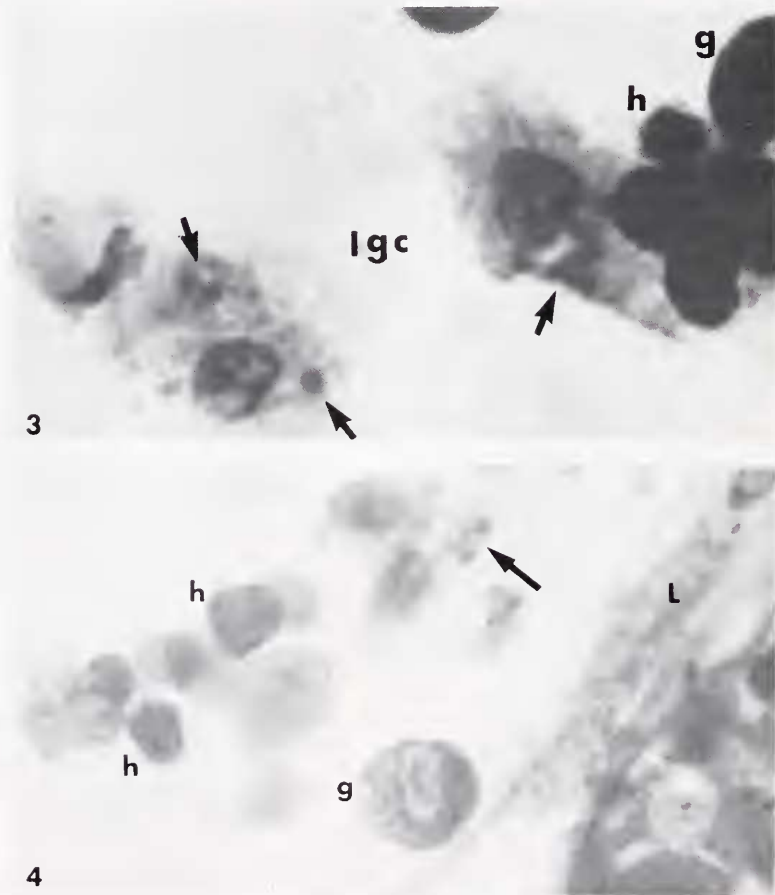


FIGURE 3. Small aggregates of hyaline cells (h) occur in the hemocoel of a crab fixed five hours postecdysis in buffered glutaraldehyde-salt. Granulocytes (g) are rare. Two large, flattened hemocytes exhibit an extensive and faintly basophilic cytoplasm which contains numerous swollen granules. Intensely eosinophilic, smaller granules surround a reticulate nucleus; peripheral granules are larger and slightly basophilic (arrows). These hemocytes have been identified as large granular cells (lgc's). Az-Eo, pH 4.5.  $\times 900$ .

FIGURE 4. Hyaline cells (h) and granulocytes (g) accumulate within the hemocoel beneath the epidermis underlying the newly formed cuticle of a crab fixed between 10 and 15 hours postecdysis in buffered formalin-salt. The granulocyte contains histidine in the cytoplasm and granules. The small hyaline cells possess a diphenol; a few appeared unstained as if they had released their phenolic contents. The arrow points to a small granular cell which contains swollen proteinaceous granules of undetermined function. A leucophore (L) passes across the field at right beneath the epidermis. DAS-AzA.  $\times 900$ .

*Fluctuations in the number of hemocytes during the molting cycle*

To determine whether fluctuations occurred in the number of hemocytes during the molting cycle, counts were made in the tissue sections taken from each tissue block. The sections were examined microscopically using low magnification (100 $\times$ ) for an area heavily populated with hemocytes. Using high magnification (450 $\times$ ) two counts were made of the hemocytes in that area.

The granulocytes and hyaline cells were counted; their relative numbers varied with the stages of the molting cycle. During intermolt and proecdysis, there were twice as many granulocytes as hyaline cells. At ecdysis, the numbers of both granulocytes and hyaline cells increased: two-fold and ten-fold, respectively. Thus, the proportion of granulocytes and hyaline cells (2/5) was the inverse of that in the earlier two stages. By 5–10 hours postecdysis, the hyaline cells outnumbered the granulocytes by 10:1. However, 24–48 hours postecdysis, the numbers of both types of hemocyte gradually declined. The decline was more severe among the hyaline cells, which still prevailed over granulocytes by 2:1 by 24 hours postecdysis.

During intermolt and proecdysis, the hemocytes were usually floating freely in the hemocoel. At ecdysis and throughout postecdysis, numerous hemocytes aggregated beneath the epidermal cells and penetrated the epidermal layer, approaching the newly formed cuticle (Fig. 1). In sections of crabs fixed at ecdysis and during early (5–10 h) postecdysis, numerous hyaline cells were packed together into large nodules floating near the epidermis, or occasionally freely within the hemocoel. In some specimens, small aggregates were formed near the epidermis by hyaline cells surrounding an occasional granulocyte (Fig. 3). Among the small aggregates, a fourth type of hemocyte could be identified as a large granular cell (Fig. 3). The large granular cells contained two types of swollen granules within a flattened faintly-basophilic cytoplasm; pale acidophilic granules encircled the nucleus, whereas basophilic granules populated the extensive peripheral cytoplasm. The pale basophilic nucleus had a reticulate chromatin network and contained an intensely basophilic nucleolus.

*Histochemical observations—the hemocytes and the serum*

*Basophilia, acidophilia, glycogen, 1,2-glycols, lipids, and aldehydes.* The basophilic staining of the hyaline cell cytoplasm became extinguished at pH 3 (Table I). At this pH the granules within granulocytes stained metachromatically; granule staining became abolished at pH 2 and basophilic nuclei and melanophore granules (still apparent at pH 1) could be visualized.

By PAS staining, the granulocytes contained 1,2-glycols which concentrated within the granules; the cytoplasm reacted moderately. By contrast, the hyaline cells appeared negative. The serum showed transient reactions which varied with the stages of the molting cycle: during early postecdysis the serum became filled with 1,2-glycols and numerous intensely PAS-positive granules like those in the granulocytes. Intensely PAS-positive granules also appeared within the epidermal cells and tegumental glands during this period. At the other stages of the molting cycle, the serum, epidermal cells, and glands became devoid of the presumed glycoprotein(s).

Fixation of crabs in chloroform:methanol freed the tissues of lipids, but no change occurred in the PAS reactions of the granulocytes (cytoplasm and granules) or the "serum granules." The serum exhibited reduced staining by PAS which could be ascribed to extracted lipids, but no sudanophilia could be demonstrated. Further proof that PAS stained non-lipid substances was obtained when acetylation abolished

TABLE I

Summary of histochemical reactions in the hemocytes, serum, cuticle, and melanophores of the fiddler crab\*

Cell or tissue component	Histochemical reactions									Azo-Serotonin
	Basophililia	Acidophililia	Stain extinction	Glycogen	1,2-Glycols	Lipids	Induced aldehydes	Native aldehydes	Proteins	
Hyaline cell	+	-	pH 3	-	-	-	± to -	-	±	-
Granulocyte	-	+	pH 2	+	+	±	++	± to -	++ to -	-
Serum	-	+	>pH 3	±	±	±	±	-	± to - (hard) + (soft)	-
Exocuticle										
hard	+	+ (endo only)	>pH 3	-	-	-	-	-	± to ±	-
soft	-	-	>pH 3	-	±	-	-	-	+	-
Melanophore granules	±	-	pH 1	-	-	-	-	-	++	+

\* Note: The words "hard" and "soft" refer roughly to the state of the cuticle during the molting cycle. "Exocuticle" was taken as representative for histochemical changes also occurring in the endocuticle (endo) during the molting cycle which often appear in parallel but at different times. Results were recorded separately when a difference in staining capacity was noted. The symbols indicate strength of the histochemical reaction: ++, intensely positive; +, positive; ±, moderately positive; ±, mildly positive; -, negative.

the reactions in the granulocytes, some of the serum granules, and reduced the PAS reaction in the serum itself; deacetylation partially restored the reactions. Digestion of glycogen from the tissue sections did not change the PAS reactions within the granulocytes. However, some of the "serum granules" showed reduced staining and therefore contained glycogen.

The induced aldehyde groups detected by black Bauer and black periodic techniques were intensely visualized within the granulocytes. In contrast, the hyaline cells reacted mildly or sometimes not at all.

Native (free) aldehydes were detectable (by direct application of Schiff reagent, 1 hour, to the tissue sections) in granulocyte cytoplasm, especially the perinuclear region, during intermolt, proecdysis and late postecdysis, but not during early postecdysis. The intracellular granules did not stain. Likewise, the hyaline cells and serum did not react.

*Diazotization reactions for aromatic end-groups.* The aromatic end-groups of proteins azo-coupled intensely (diazosafranin pH 7.8) within the granulocytes during most of the molting cycle (except late postecdysis) but only mildly in the hyaline cells. Interestingly at 48 hours postecdysis the granulocytes lost the ability to azo-couple as if they had released the responsible proteins. During this period, the serum showed increased reactions as if it had received the proteins released from the granulocytes. However, during proecdysis the serum must not have contained these proteins because it did not react. Subsequent extraction of the azo-coupled tissue sections in

TABLE I (Continued)

coupling		Protein End-Groups					
Catechol- amines	Histi- dine	Tryptophan	Arginine	Tyro- sine	Amino	Reducing substances	Copper
++ to -	-	± to -	-	±	±	- (hard) ± (soft)	±
-	+ to ++	+ to ±	±	++	++	- (hard) + (soft)	++ (hard) + (soft)
± to - (hard) + (soft)	±	± (hard) + to ± (soft)	-	±	± to ± (hard) ± to - (soft)	+ to -	+ to ± (hard) ± (soft)
- ++ to ±	±	- ± to -	- ± to -	- ±	- ++ to ±	± to - ++ to +	± + to ±
- (cytoplasm ++)	+	+	-	-	-	+ (hard) ++ (soft) (cytoplasm +)	-

dilute HCl failed to remove the tightly bound dye. Serotonin could not be detected using diazosafranin, pH 3.2 (Lillie *et al.*, 1973).

By azo-coupling with DAS-AzA, a primary catecholamine was demonstrated within the hyaline cells (Lillie *et al.*, 1973b, c) during most stages of the molting cycle (Fig. 4). During postecdysis, the phenolic substance gradually disappeared. Early in postecdysis, intact hyaline cells near or within the epidermal net azo-coupled mildly, as if they were losing their former contents. A phenol visualized in the serum during intermolt and proecdysis was still detectable early in postecdysis. However, by 48 hours postecdysis, the phenol in the serum became substantially reduced, and also disappeared from the hyaline cells.

Using DAS-AzA, two additional granule-containing hemocytes could be identified: small granular cells the size of hyaline cells (Fig. 4), and the large granular cells previously identified by Az-Eo (Fig. 3). The small and large granular cells contained swollen granules which exhibited intense azo-coupling (Figs. 4, 5). The large granular cells increased their numbers during early postecdysis (Fig. 5A) when two forms became apparent: cellular forms possessed a distinct cell shape and a nucleus (Fig. 5B); amorphous forms had a more extended cytoplasm and no nucleus (Fig. 5C). By 15-24 hours postecdysis, the large granular cells aligned along the epidermis (Figs. 6A, B). Morphologically they resembled melanophores, except they contained larger (swollen) granules.

Intermediate ("transitional") cells, fixed in the process of rupturing, spewed forth from their cytoplasm numerous tiny granules which azo-coupled with DAS-AzA (Fig. 2B, C). These granules approximated the size of bacteria. However, prior extraction with KOH did not remove azo-coupling capacity. Therefore, RNA was not responsible. Furthermore, no gram-positive material was demonstrable. Epidermal melanophore granules also azo-coupled intensely (Fig. 6C). Surrounding them, the cytoplasm of the melanophores azo-coupled as if it contained a phenol. The sites of azo-coupling



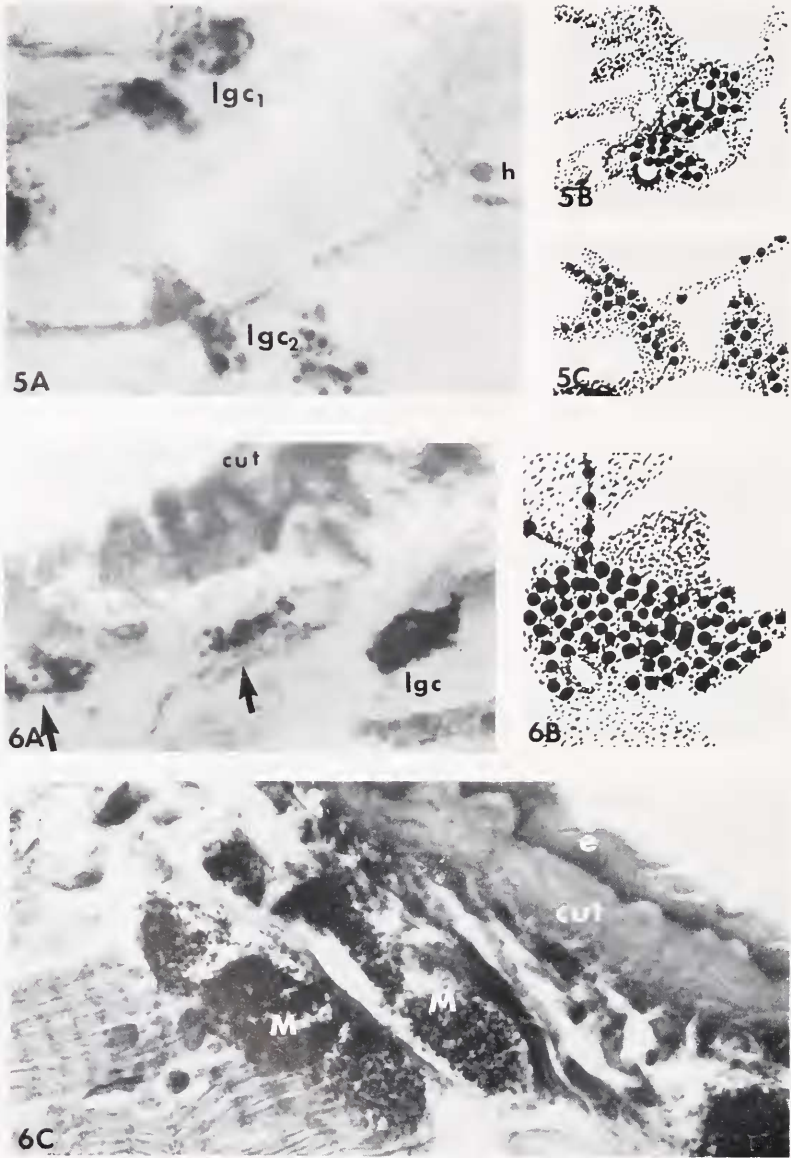


FIGURE 5. The number of large granular cells ( $lgc$ ) increases in the hemocoel, especially near the epidermis, after ecdysis. The specimen was fixed 10–15 hours postecdysis in buffered formalin-salt. Figure 5A shows two types of large granular cells: type 1 ( $lgc_1$ ) possesses discrete cytoplasmic boundaries and a nucleus; type 2 ( $lgc_2$ ) has an amorphous cytoplasm and no nucleus can be seen. Both types contain characteristic swollen granules whose protein matrix has azo-coupled with DAS-AzA. The hyaline cell (h) is much smaller and contains diphenols. Figures 5B and 5C show diagrammatic interpretations of the two types of large granular cells shown in Figure 5A. Figure 5B shows the distinct cellular shape of an  $lgc_1$ . Large swollen granules (dark circles) surround the nucleus (clear space). Figure 5C shows the amorphous cytoplasm of an  $lgc_2$  which also contains swollen granules (dark circles). No nucleus can be seen perhaps indicating that these large granular cells are degenerating and releasing their contents into the serum. DAS-AzA.  $\times 600$ .

FIGURE 6. The relationship between the large granular hemocytes and melanophores is uncertain. Figure 6A shows three large granular cells ( $lgc$ ) near the epidermis of a crab fixed 5–10 hours postecdysis

described above could not be decolorized by prolonged extraction with dilute HCl.

*Verification of a diphenol: oxidation-reduction experiment.* After prior oxidation, the phenols detected within the hyaline cells and serum converted into quinones and could not azo-couple with DAS-AzA. Pretreatment with dithionite reduced quinones into phenols which could then azo-couple. After brief, 4 hours, dithionite treatment, the suspected phenols in the hyaline cells and the serum curiously azo-coupled less intensely; however, prolonged dithionite (8 h) treatment rendered the staining more intense at both sites. When dithionite-reduced tissue sections were oxidized, diphenols became visualized again in the serum and hyaline cells. The intracellular granules described by DAS-AzA within the small and large granular cells, and intermediate cells remained unaffected by oxidation or reduction. Often the reducing solution extracted the granules from epidermal melanophores; the azo-coupled cytoplasm, unaffected by reduction, was rendered negative by oxidation, verifying its phenolic content.

*Demonstration of histidine and amino groups.* The color of the granulocytes (cytoplasm and granules) and some of the serum granules after DAS-AzA (Fig. 4) resembled that of erythrocytes containing histidine (Lillie *et al.*, 1973b, c). Pretreatment with  $\text{FeCl}_2$  blocked the reaction, confirming the presence of histidine (Lillie *et al.*, 1971). Oxidation and reduction rendered the sites more intense. Increased histidine reactions occurred in the granulocyte and serum granules during intermolt, proecdysis, and early postecdysis. By this and other reactions (PAS, diazosafranin), some of the serum granules may be identical to (and released from) the granulocytes during the molting cycle.

Tryptophan (PCB reaction) appeared in the granulocytes during early postecdysis. However, by late postecdysis, the reaction decreased as if the cells released their proteinaceous contents. Like the granulocytes, the serum became positive during early postecdysis, but reacted less intensely 24 hours postecdysis, as well as during intermolt and proecdysis. The hyaline cells reacted mildly, or not at all, throughout the molting cycle.

Small amounts of arginine (NQS reaction, Fig. 1B) and large amounts of tyrosine could be visualized within the granulocytes during all stages of the molting cycle. In contrast the hyaline cells and serum exhibited reactions which were negative for arginine and mild for tyrosine at all times. Pretreatment of tissue sections with tetranitromethane selectively abolished the staining for tyrosine.

The presence of amino groups (with and without prior deamination) was verified by the uptake of iron (FeII) subsequently visualized by hematoxylin or by acidophilia (Az-Eo). The granulocytes stained intensely; their staining could be abolished by prior deamination. Amino groups visualized in the serum during intermolt and proecdysis were deaminated inconsistently; their presence varied during postecdysis, possibly indicating the disappearance of a protein during this time. Hyaline cells showed mild reactions at all times.

*Reactions for reducing sites.* The hyaline cells, granulocytes, and serum showed cyclic reactions for reducing substances during the molting cycle. Minimal at ecdysis, the reducing substances could not be visualized within hyaline cells at any other time.

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in buffered formalin-salt. A type 1 large granular cell (an intact cell) with nucleus is shown at the right with the label lgc; type 2 large granular cells (amorphous without nucleus) appear at arrows to left.  $\times 600$ . Figure 6B diagrammatically depicts at higher magnification the type 1 large granular cell shown in Figure 6A.  $\times 900$ . Figure 6C shows mature melanophores (M) near epidermis and cuticle of a crab fixed 10 hours postecdysis in buffered formalin-salt. Diphenols azo-couple in the exo- (e) and endocuticle (cut), and are present in the cytoplasm of the melanophore. The melanophore granules are also proteinaceous but tiny compared with the swollen granules in the large granular cells in Figure 6A. DAS-AzA.  $\times 600$ .

Granulocytes remained positive between ecdysis and 5 hours postecdysis; but also became negative during the later stages of postecdysis. During late postecdysis, the serum contained reducing substances (released from the granulocytes?), but lost them cyclically during early postecdysis, intermolt, and proecdysis. The tegumental glands also reacted strongly during postecdysis. The reducing substances described above were visualized by ferricyanide, Clara's hematoxylin, and Fell acid ferrocyanide reactions.

With ferric ferricyanide, positive sites also appeared within the granules of small and large granular cells, intermediate cells, and the serum (including large swollen granules). The intracellular granules and the swollen serum granules were not affected by oxidation or reduction, and therefore they do not contain reducing groups. The serum granules having the size of those within granulocytes were affected by oxidation and reduction.

Melanophores contained numerous small granules which blackened characteristically in ammoniacal silver after 10 minutes and stained intensely with ferricyanide. The granules were contained by a positive cytoplasm. Oxidation intensified the ferricyanide (quinhydrone) reaction in the granules and masked the visualization of reducing phenols in the cytoplasm. Reduction restored the original reactions, and enabled the visualization of reducing substances (some probably phenols) within the formerly-negative hyaline cells, granulocytes, and serum. Oxidation rendered the sites negative once again.

Surprisingly, epidermal melanophore granules intensified their natural brown color by incubation in Clara's solution during postecdysis, but not during proecdysis. Reducing substances may be responsible for the transient reaction.

*Copper-rich sites.* Identification of copper-containing sites failed using ammoniacal rubeanic acid. Using Clara's hematoxylin, copper could be visualized mildly in the hyaline cells: more intensely in the granulocytes. In copper uptake studies, the hemocytes took up copper to a moderate degree. The reactions of the granulocytes and serum varied with the molting cycle: during postecdysis, the granulocytes stained intensely. The serum took up copper during intermolt, proecdysis, and late postecdysis. However, during early postecdysis, the serum itself showed weaker copper uptake; but intensely positive serum granules (released from the granulocytes?) could be seen.

### *Histochemical observations—the cuticle*

*Basophilia, acidophilia, glycogen, 1,2-glycols, lipids, aldehydes, and azo-coupling.* Using the various histochemical procedures described above, we recorded the changes in the staining of the cuticle during the molting cycle. For the structure of the exoskeleton, Skinner's (1962) terminology was applied. An upper thin lipid epicuticle was distinguished from the procuticle below. The procuticle was divided into an upper thin exocuticle (pigmented layer) and, beneath it a thicker endocuticle (calcified layer). Epi- and exocuticle form during proecdysis; and endocuticle during postecdysis (Skinner, 1962). During late postecdysis, a deeper layer forms over the epidermis which is thin and uncalcified called the membranous (uncalcified) layer, and which does not undergo further modification by calcification or quinone tanning. In this study, the membranous layer was rarely seen.

Curiously, the exocuticle of freshly molted crabs did not stain with Az-Eo. However, by late postecdysis, the exocuticle and underlying endocuticle attained a weak basophilia. Characterizing the influx of acidic substances, this basophilia increased as the entire procuticle became wider and hardened. The new endocuticle, formed by 24 hours postecdysis, remained unstained by Az-Eo up to 48 hours postecdysis. Later,



when more fully formed and hardened, it became acidophilic, as if basic substances had penetrated.

The epicuticle which covered the fully tanned exocuticle exhibited basophilia. However, at ecdysis, the epicuticle became acidophilic. It contained aldehydes by the black Bauer and black periodic methods; sudanophilia was absent.

By PAS staining, the new exocuticle contained 1,2-glycols variably during proecdysis, ecdysis, and late postecdysis. This reaction became abolished by acetylation, but it was not restored by deacetylation. Exo- and endocuticle did not react early in postecdysis. The reaction was negative in intermolt crabs. No glycogen, aldehydes, nor lipids were detected.

Using various diazonium salts, the cyclic appearance of aromatic protein end-groups was detected within the cuticle's protein matrix. The exocuticle layer azo-coupled mildly during proecdysis and ecdysis indicating proteins exist in low concentration. The azo-coupling of the cuticle proteins intensified during early postecdysis. During late postecdysis, more aromatic end groups appeared in the outermost exocuticle than in the newer endocuticle layer. The epicuticle did not azo-couple. Subsequent extraction in weak HCl failed to change the results.

*Diphenols in cuticle.* The reaction of the cuticle to the DAS-AzA showed variations in the staining for phenols throughout the molting cycle. At intermolt and proecdysis, the fully-formed and hardened (quinonized) procuticle did not azo-couple. In contrast, phenols penetrated the soft exocuticle at ecdysis and azo-coupled intensely (Fig. 7A). The azo-coupling capacity of phenols in the exocuticle decreased as tanning progressed during early postecdysis; by 10 hours postecdysis only small amounts could be detected. By late postecdysis, the new endocuticle still reacted moderately for phenols. However, its azo-coupling capacity continued to decrease during late postecdysis as the width and hardening of the procuticle increased (Fig. 7B). The epicuticle showed intense reactions for phenols during the entire molting cycle (Fig. 7B).

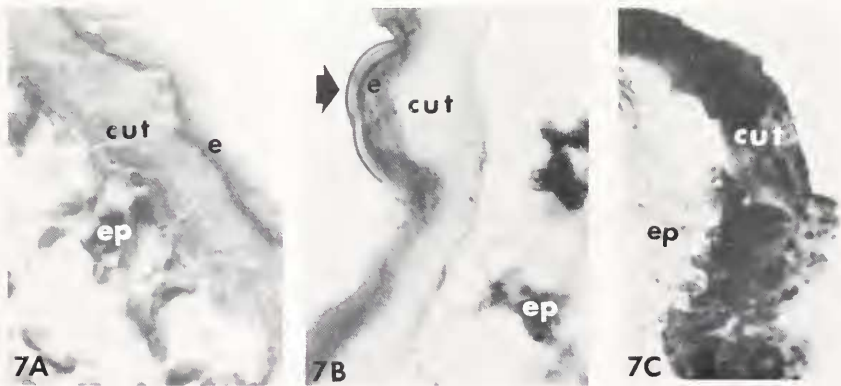


FIGURE 7. The cuticle changes its reaction for diphenols before and after tanning takes place. Figure 7A shows the intense azo-coupling of phenols in the soft exo- (e) and endocuticle (cut) of a crab fixed at ecdysis in buffered formalin-salt. Both the exo- and endocuticle react. Phenol-laden hyaline cells and histidine-rich granulocytes (not shown) occur in the hemocoel and gather close to the epidermal cells (ep) which also contain phenols during this time. Figure 7B shows the nonreactive tanned endocuticle (cut) of a crab fixed in formalin-acetic acid-salt 24-48 hours postecdysis. Exocuticle (e) reacts only mildly. Epidermal cells (ep) still react at this time. Waxy epicuticle (arrowhead) azo-couples throughout the molting cycle. DAS-AzA. Figure 7C shows the reducing capacity of the soft cuticle (cut) after fixation in Barnett-Bourne silver solution. The cuticle loses its reducing capacity as it tans. Reducing substances (non-phenolic) also appear in granulocytes; hyaline cells contain phenols which react mildly or not at all. Epidermal cells (ep) are negative.  $\times 600$ .



Temporally, the azo-coupling of the phenols in the endocuticle paralleled that in the older exocuticle, but occurred at later times. If the endocuticle began to form soon after ecdysis, its azo-coupling capacity coincided closely with that of the exocuticle layer (Fig. 7A). During late postecdysis, the endocuticle became non-reactive prior to the older exocuticle above it (Fig. 7B). The reactions probably depend on the extent to which phenols penetrate and become quinonized during tanning.

Oxidation and reduction procedures verified the presence of a diphenol within the cuticle. After oxidation, the diphenol in the cuticle became quinonized and no longer reacted. After reduction (4 h), tanned cuticles which did not azo-couple with DAS-AzA exhibited the presence of a diphenol.

*Demonstration of histidine and amino group.* Tryptophan and arginine could not be detected during proecdysis and ecdysis, although sometimes the exocuticle of newly molted crabs showed a mild reaction for arginine. Small amounts of tyrosine (selectively abolished by tetranitromethane) were detected in the exocuticle and epicuticle, but not in the endocuticle.

Prior incubation in  $\text{FeCl}_2$  reduced the DAS-AzA reaction in the cuticle, indicating the presence of histidine. The presence of amino groups in cuticle was verified by  $\text{FeII}$  uptake stained by hematoxylin or Az-Eo, with and without prior deamination. Amino groups stained intensely but sporadically in the exocuticle between ecdysis and 10 hours postecdysis; the endocuticle reacted less intensely. The data show that histochemical changes in the cuticle occur early in postecdysis, and imply that protein(s) penetrate at this time.

*Reducing substances in cuticle.* Short (10 min) incubations in ammoniacal silver gave no reaction in the cuticle; after 24 hours, reducing sites (possibly phenols) became moderately visible. No reaction occurred in acid silver. Interestingly, the cuticle of crabs fixed during ecdysis and early postecdysis in Barnett-Bourne solution strongly reduced silver (Fig. 7C). Fully formed and hardened cuticle of intermolt exhibited the mild reduction of silver.

The epicuticle contained substances which reduced silver during postecdysis, but not at other times in the molting cycle. Curiously, no reducing substances were detected in the epicuticle with ferric ferricyanide.

With ferricyanide, reducing substances in the cuticle varied cyclically with the molting cycle. During intermolt and proecdysis, the hardened cuticle did not react. In contrast, reducing substances penetrated the new exocuticle at ecdysis and reacted intensely; the endocuticle reacted less intensely. The reducing substances were not detected during postecdysis. Reduction reversed the results obtained in the negative (quinonized) cuticles of specimens fixed during postecdysis and visualized reducing substances. However, no change was induced within the fully-quinonized cuticles of intermolt, proecdysis, and late postecdysis. Oxidation of reducing substances present in the cuticle at ecdysis rendered them negative.

Oxidizing substances (visualized by  $\text{FeII}$ -acid ferricyanide) were mildly or not detectable in the endocuticle and the epicuticle. The exocuticle reacted intensely.

Using Clara's hematoxylin, copper was moderately visualized in the fully-formed, hardened intermolt cuticle. During postecdysis, the visualization of copper in the exocuticle decreased continuously. Curiously, intense amounts of copper were seen in the endocuticle during late postecdysis. The epicuticle did not react.

## DISCUSSION

By histochemistry and morphology, the present report identifies five types of hemocytes in the hemocoel of the fiddler crab. The two most commonly encountered types are a small agranular hyaline cell, or cystocyte, characterized by a scanty basophilic

cytoplasm encircling a densely basophilic nucleus; and a larger granulocyte containing numerous eosinophilic granules within an eosinophilic cytoplasm. The other three hemocytes were: an intermediate cell, partially resembling the hyaline cell and the granulocyte, and thus appearing to be a transitional stage in the granulocyte maturation process (Toney, 1958; Ravindranath, 1980); a small granular cell, and a large granular cell. Perhaps the latter two also represent transitional stages in the granulocyte maturation process (see Ravindranath, 1980, for review). However, their functions are unknown.

The granulocytes of several arthropod species transform their shape and degranulate on exposure to air into intermediate cells and hyaline cells (Wharton Jones, 1846; Hardy, 1892; Vranckx and Durliat, 1977). Degranulation after swelling has been associated with clotting in *Limulus* and *Homarus* (Dumont *et al.* 1966; Hearing, 1969). In vertebrates, degranulation may result from cell injury, autolysis, aging, death (Deruby, 1918; Myers and Dewolf-Glade, 1964). The present data show evidence for degranulation of intermediate cells (Figs. 2B, C), and large granular cells (Figs. 5A, C) and include indirect data for degranulation of small granular cells (personal observations) and granulocytes (histochemically by their resemblance to serum granules).

The hyaline cells and the granulocytes of *Uca pugilator* may be involved in tanning at certain points in the molting cycle. Counts of the numbers of hemocytes show cyclic events occur and verify earlier work that both granulocytes and hyaline cells increase their numbers to a peak at ecdysis (Kollman, 1908). The present report indicates the granulocytes predominate before ecdysis; the hyaline cells after ecdysis. Histochemically, these hemocytes cyclically contain protein end groups and diphenols respectively which seem to be shuttled into the serum and new exocuticle at ecdysis.

Biochemically, the blood appears to be the main site of tanning agent synthesis. Using paper chromatography, Vacca and Fingerman (1975a) identified N-acetyldopamine and N-acetylnoradrenalin as metabolites of labeled dopamine (as well as their beta-glucosides) which appear in the blood of the fiddler crab, *Uca pugilator*, during ecdysis. Subsequent incorporation into the cuticle suggests the N-acetylated dopamine metabolites attach to the glucosides and then act as tanning agents. Prior to cuticle incorporation, they become attached to two large blood proteins (>400,000 d and ~150,000 d) which transport the tanning agents into the soft cuticle. The appearance of free glucosides and attached carrier proteins in the blood is cyclic and corresponds to the incorporation of label into the cuticle during postecdysis (Vacca and Fingerman, 1975b).

Histochemically, the diphenolic substance(s) visualized in the hyaline cells at ecdysis and early postecdysis, when the hyaline cells occur in large numbers, may represent the tanning agent(s) or precursor(s). Probably a primary catecholamine, candidate tanning diphenols include norepinephrine (as demonstrated histochemically in the adrenal medulla by Lillie *et al.*, 1973a), DOPA, dopamine, N-acetyldopamine, and N-acetylnorepinephrine. Interestingly, the hyaline cells lose the diphenol during late postecdysis, as the new cuticle tans. They appear in large numbers near the epidermis, looking empty as if their contents had been released. Like the hyaline cell, the serum contains a phenol during intermolt, proecdysis, and early postecdysis. However, by 48 hours postecdysis, its presence becomes diminished. Speculatively, the diphenol in the serum originates from the hyaline cells, and enters the soft cuticle during ecdysis and early postecdysis.

During late postecdysis, the diphenol cannot be visualized in the hardened exocuticle without reduction by dithionite. Presumably, the diphenol acts as a tanning agent and cross-links with the cuticle protein matrix, transforming into the non-reactive quinone form during late postecdysis, intermolt, and proecdysis.

Vacca and Fingerman (1975b) speculated that a permeability factor enables the

rapid transfer of tanning agents from the blood (hemocytes and serum) into the cuticle during early ecdysis. Precedence for the hormonal control of tanning comes from insect studies: ecdysone and ecdysterone accelerate the formation of dopamine from precursor tyrosine within the hemocytes of tsetse fly puparia *in vitro* (Whitehead, 1971); bursicon stimulates hemocyte permeability in the initial stages of tanning agent synthesis, thereby enabling them to overcome a concentration barrier to tyrosine (Whitehead, 1970). Bursicon also stimulates lysine uptake by the cuticle (Fogal and Fraenkel, 1969). The diuretic hormone of the American cockroach enables the removal of excess liquid from the blood (*via* Malpighian tubules) during postecdysis, and also enhances the uptake of compounds such as tyrosine by the hemocytes and epidermal cells (Mills and Whitehead, 1970). Among the crustaceans, ecdysone triggers protein synthesis within the hemocytes of the crayfish *Orconectes limosus* during proecdysis (Keller and Adelung, 1970).

Various histochemical procedures visualize proteins, amino end groups, and amino acids within the granulocytes. Cyclic histochemical reactions imply that these hemocytes serve in the production of proteins during the molting cycle. Some of these groups (arginine and tyrosine, lysine and histidine) occur in the granulocytes (cytoplasm and granules) throughout the molting cycle, and can be visualized as structural elements of the protein matrix of the cuticle and the granules of the granulocytes. Other end groups appear cyclically: tryptophan (also appearing in serum) became visible in the granulocytes and serum during early postecdysis. Although detectable in the cuticle matrix throughout the molting cycle, lysine, histidine, and aromatic protein end-groups become histochemically intense and probably enter the cuticle during early postecdysis when it is still soft. Indeed, water-soluble proteins extracted from insect cuticle exhibit the free end groups of lysine; but the same groups cannot be demonstrated in sclerotized cuticle (Hackman, 1953). The visualization of additional protein end-groups in the cuticle may represent the incorporated protein carriers detected biochemically (Vacca and Fingerman, 1975a, b). The tanning protein in the hemolymph of the insect *Manduca sexta* is immunologically identical to cuticle protein (Koeppel and Gilbert, 1973). Unfortunately, the precise relationship between the cuticle protein matrix of *Uca* and the proteins carried by the granulocytes cannot be precisely determined from the present data.

The granules of the granulocytes contain basic (amino) end groups (lysine, arginine, and histidine). The reactions in serum suggest that these granule constituents are released after ecdysis as well. As the new exoskeleton forms, numerous free granules appear in the serum thereby encouraging the speculation that the granulocytes release their proteinaceous granules, as well as a cytoplasmic protein, into the serum during early postecdysis. By 48 hours postecdysis, the release process seems to be complete. Diverse serum granules were detected histochemically in *Uca* and have been reported in other arthropod hemocytes by histochemical and ultrastructural studies (see Ravindranath, 1980, for review). The different granules may represent stages in the coagulation process (Ravindranath, 1980), or may possess different functions including basement membrane formation, wound healing (Ravindranath, 1980), or tanning. The diverse functions may account for some of the staining variations of granules visualized in this report within the granulocytes, large and small granular cells, and "serum granules."

Granulocytes which contain reducing substances (probably non-phenolic) during most of the molting cycle, become unreactive at ecdysis and 5 hours later, as if their reducing substances become released. Deep within the hemocoel, small, perhaps immature, granulocytes still react intensely (Fig. 1C). The data coincide with the synthesis and release of a weakly acidic glycoprotein (perhaps a carrier which contains sulfhydryl or other reducing groups) during early postecdysis; alternatively, protein synthesis



becomes blocked or breakdown increases. Minute amounts of native aldehyde detectable within the granulocytes during intermolt, proecdysis, and late postecdysis also disappear during early postecdysis.

Surprisingly, serum (apart from its contained granules) contains few soluble reducing substances during postecdysis, when biochemically it sequesters both tanning phenols and protein carriers (Vacca and Fingerman, 1975a). Perhaps the weak histochemical reaction reflects their transient presence; alternatively these substances are not detectable because they are bound to glucosides (Vacca and Fingerman, 1975a) or to the granules released from the granulocytes.

In other arthropod species, hemolymph proteins appear cyclically during the molting cycle. *Carcinus* blood contains a glycoprotein throughout the molting cycle which disappears at ecdysis and then reappears 10 days later (Busselen, 1970), the time during which sclerotization is complete in *Uca*. Its appearance and maintenance depends upon the nutritional status of the organism. *Gecarinus* also possesses a blood protein involved in clotting which becomes barely detectable during postecdysis (Strutman and Dolliver, 1968). The present study shows that intermediate cells rupture easily and spill their proteinaceous granules into the serum during early postecdysis. These cells may be involved in clotting. Curiously, the ability of the blood to clot is minimal during postecdysis (Strutman and Dolliver, 1968). A noteworthy speculation as to why the animal is at such a disadvantage when it is most susceptible to injury and infection might be that most of the hemocytes instead become involved in the synthesis of other substances (proteins and diphenols) to be used for sclerotization. Indeed, this function would take priority in order to reinstate the animal into its protective shell after growth.

The mechanism of hemocyte degeneration may play a significant role in supplying tanning agents to the cuticle. By late postecdysis, the serum and hyaline cells of *Uca* become exhausted of diphenols. With the loss of their diphenols, few hyaline cells remain intact and their numbers diminish severely. In addition, granulocytes release their granules and reduce in number during early postecdysis. Hypothetical tanning hormone(s) might increase hemocyte permeability to substances, thereby causing swelling and eventual lysis. This mechanism could account for the numerous granules visualized in the serum during postecdysis, and has been proposed for the numerical decrease in the hemocytes during postecdysis (Marrec, 1944). Histochemical and ultrastructural evidence exists for the disintegration and vesiculation of lipoprotein cells and nuclei with subsequent streaming-in of neighboring hemocytes during proecdysis and postecdysis in the crab *Paratelphusa* (Adiyodi and Adiyodi, 1972). Cell explosion of hyaline cells and granule release by intermediate cells and granulocytes have been postulated as mechanisms of clotting (Hardy, 1892; Tait and Gunn, 1918; Wood *et al.*, 1971; see review by Ravindranath, 1980), and tyrosinase liberation (Pinhey, 1930).

Based on his *in vitro* studies, Summers (1968) proposed that the epidermis, not the blood, is the site of tanning agent synthesis. We now present evidence that diphenols (presumably tanning agents) appear in the epidermis transiently between ecdysis and early postecdysis. These data, as well as previous biochemical evidence (Vacca and Fingerman, 1975b), suggest that the epidermis is a site of translocation rather than synthesis. Degeneration as a mechanism of tanning would cause the hemocytes to release their tyrosine-metabolizing enzymes, and would account for Summer's findings that most of the tyrosinase enzyme activity occurs in the plasma, and not the hemocytes, of the fiddler crab (Summers, 1967).

Histochemically, our study shows the hyaline cells in *Uca* take up copper, a component of hemocyte tyrosinase (Pinhey, 1930). Functionally, tyrosinase oxidizes phenols (tanning agents) to quinones which then act as strong oxidizing agents. Under



pathological conditions, quinones respond to injury and infection by forming melanin upon coagulation (Taylor, 1969). Therefore, despite a deficient clotting mechanism, perhaps the soft-shelled crab possesses the enzyme complex within the hemocytes and eventually the serum, during tanning as a ready system for defense. Indeed, the granulocytes also take up copper (especially during early postecdysis when the serum is least reactive), and contain a substance which can oxidize Clara's hematoxylin.

Koepe (1971) proposed that tyrosinase is the actual protein carrier of tanning agents in insects. Unfortunately, no oxidizing capacity could be detected histochemically in the serum, and though the exocuticle oxidizes FeII (by acid ferrocyanide reaction) at ecdysis, it resists copper uptake. Therefore, we propose another blood protein complex may be involved in the tanning process. The tegumental glands, known to possess tyrosinase, also oxidize FeII and may function in an aspect of tanning (Stevenson, 1963a, b) which histochemically relates to the epicuticle. Indeed, multiple mechanisms may exist as implied by variable basophilia or acidophilia of exo- and endocuticle.

Interestingly, reducing sites were demonstrated in the melanophore granules at ecdysis (intensified ferricyanide reaction after oxidation). Azo-coupling reactions demonstrate the presence of a protein matrix which contains amines, diphenols, and indoles. After ecdysis, these histochemical reactions changed. The data imply that amine and phenolic (tanning?) substances enter a protein-granule matrix within the melanophores during ecdysis, when tanning agents and permeability factors are available. Indeed melanin may form at the end of the tanning process as the result of a biochemical equilibrium displacement of tanning agent biosynthesis.

Histochemically, the epidermal melanophore granules resemble melanin by their argentophilia, strong basophilia, ability to take up iron, insolubility in organic solvents, and their negative PAS, acid fast, and lipid reactions. However, Noël (1982) reports that *Uca* melanophore pigment is ommochrome. Indeed the present study does not define the granules as melanin. Dithionite used for reduction extracted many melanophore granules; those which remained could not be reduced even after 8 hours.

The epidermal melanophores exhibited the presence of a cytoplasmic diphenol throughout the molting cycle. Unlike the melanophore granules, perhaps the cytoplasm maintains a separate pool of diphenols in continuous supply. It is tempting to speculate that the production of diphenolic tanning agents within the hemocytes might relate biochemically to the process of pigment formation.

Indeed certain histochemical reactions of the granules visualized in the small and large granular cells resembled those of the melanophore granules. The appearance of numerous large granular cells near the epidermis during early postecdysis suggests a relationship with melanophore formation, which has not yet been defined.

Interestingly, the large granular cells azo-couple (DAS-AzA) only during ecdysis and postecdysis. Apparently, they sequester tanning agents from the blood and deposit them on the protein matrix of the contained swollen granules. In this way, the large granular cells, like the melanophores, may function in the disposal of excess tanning agents remaining in the blood during postecdysis. This mechanism of disposal provides an alternative to glucoside formation which masks the tanning agents prior to use (Vacca and Fingerman, 1975a, b).

In conclusion, these histochemical data parallel previous biochemical findings which document the cyclic appearance of a protein-bound phenolic tanning agent in the blood of *Uca pugilator* (Vacca and Fingerman, 1975a, b). We now show that granulocytes contain protein end groups in their cytoplasm and granules which might represent portions of the protein carrier complex: the hyaline cells contain diphenols which could act as tanning agents. The reducing groups of proteins and diphenols

become demonstrable in the cuticle during the early postecdysis and cannot be visualized by 10 hours postecdysis when tanning (quinonization) takes place. Additionally, the cyclic presence of proteins and diphenols in the blood cells, serum, and cuticle indicates the existence of a tanning hormone (perhaps more than one) which enables the cyclic synthesis of the diphenolic tanning agents and protein carriers, and appropriately shuttles them from the hemocytes into the serum and cuticle during the molting cycle. Fingerman and Yamamoto (1964) provided evidence that tanning of the cuticle of the dwarf crayfish, *Cambarellus shufeldti*, is hormonally controlled. However, the tanning hormone(s) remain(s) to be identified in crustaceans.

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